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(54) Title: REFINING OIL COMPOSITIONS

(57) Abstract

     This invention relates to a process for treating an oil composition containing saturated and unsaturated fatty acids in the form of triglycerides, in order to obtain a refined product with higher concentrations of the polyunsaturated fatty acids.

## REFINING OIL COMPOSITIONS

This invention relates to a novel process for treating an
oil composition containing saturated and unsaturated fatty
acids in the form of glycerides, in order to obtain a
refined product with higher concentrations of the
5  polyunsaturated fatty acids.      The invention in
particularly preferred embodiments provides a process for
increasing the concentrations of EPA and DHA in a fish oil
composition.

In this specification polyunsaturated fatty acids are
10  identified according to the system wherein the omega- or
n-number denominates the position of the first double bond
when counting from the terminal methyl group, e.g. in an
omega-3 or n-3 fatty acid, the first double bond occurs at
the third carbon carbon-bond from the terminal methyl group
15  of the acid.    Further, when a fatty acid is identified,
for instance, as C18:3, this refers to a fatty acid having
18 carbon atoms in the chain and three double bonds.

Commercially important polyunsaturated fatty acids are EPA
(eicosapentaenoic acid, C20:5) DHA (docosahexaenoic acid,
20  C22:6)    and AA (arachidonic acid, C20:4).    The full
nomenclature of these acids according to the IUPAC system
is:

     EPA
     cis-5,8,11,14,17-eicosapentaenoic acid

25    DHA
     cis-4,7,10,13,16,19-docosahexaenoic acid

     AA
     cis-5,8,11,14-eicosatetraenoic acid

As is well known, EPA and DHA are proving increasingly

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valuable in the pharmaceutical and food supplement industries in particular. These acids are found in relatively high concentrations in certain marine oils, but for many purposes it is necessary that the marine oils
5 should be refined in order to increase the content of EPA and/or DHA to suitable levels, or to reduce the concentrations of, or even eliminate, certain other substances which occur naturally in the raw oil. For pharmaceutical and food purposes, for instance, it is
10 necessary to substantially completely eliminate all the pesticide residues which commonly occur in marine oils, even those derived from fish caught in sea areas quite remote from intensively cultivated land areas.

EPA and DHA must exhibit an all-cis (Z-Z) configuration
15 corresponding to their naturally occurring state if they are to exhibit biological activity without toxicity. However, these acids are extremely fragile when they are heated and they very readily undergo fast oligomerization, isomerization and peroxidation reactions. Accordingly, it
20 is extremely difficult to purify marine oil compositions containing EPA and DHA without risking loss of these desired acids in their useful form.

EPA and DHA occur in marine oils predominantly as their triglycerides. Up until now, most practical refining
25 processes start either by esterifying the oil with a low molecular weight alcohol (normally ethanol) or by hydrolysing the oil to form free acids or their salts, whereafter fractionation of the oil to recover the desired product is initiated.

30 However, because of the complexity of marine raw materials, polyunsaturated fatty acid derivatives in highly purified form are not easily prepared by any single fractionation technique. Usually a combination of techniques is therefore used, the particular combination chosen depending

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on the composition of the raw material and the
concentration and other quality criteria that are desired
for the product.  Urea complexation is one fractionation
technique which is commonly employed in processes for
5  recovering high content EPA and/or DHA compositions.

Urea has the useful property of forming solid complexes
with straight-chain organic compounds.  When a marine oil
composition containing fatty acids or esters is added to a
solution of urea, a crystalline complex is formed with the
10  more saturated fraction of the acids.  The crystals can be
removed, leaving a raffinate of more unsaturated fatty
acids or fatty acid esters.

Urea complexation has been used with both free fatty acids,
and with methyl or ethyl esters of the fatty acids.  The
15  process can be made continuous by using heat exchangers
with a scraped surface as reactors for urea occlusion
formation.  When fractionating esters, it seems to be the
normal procedure first to react the oil with alcohol and/or
alcohol/water and then to isolate the esters/free fatty
20  acids before urea complexation.   However, a combined
in situ esterification and urea fractionation may also be
performed as described in EP-A-0255824.

When such a process is used in combination with, for
instance, two or more steps of molecular distillation, it
25  is possible to produce a refined product containing 85% by
weight or higher of n-3 polyunsaturated fatty acids,
predominantly EPA and DHA from a raw marine oil.  However,
the total recovery of the refined product is undesirably
low.    In a typical commercial operation using such
30  conventional fractionation processes, one might expect to
recover only about 60-80 tons of an 85% n-3 fatty acid
concentrate from 1000 tons of raw marine oil, i.e. a
recovery rate of only 6-8%.   This poor yield means not
only that such refining processes are very expensive but

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also that they require bulky, complex equipment.

The lipophilic character of many environmental pollutants
(examples: pesticides and polychlorinated biphenyls)
results in an accumulation of these compounds in marine
5    lipids.    Unfortunately, urea does not form complexes with
many such pollutants, and as a result a concentrate from
urea complexation will contain increased, and for many
purposes unacceptably high, levels of pesticides and other
environmental pollution products as compared with the
10   original marine oil.    Consequently, current refining
processes based on urea complexation for making refined
fish oils for human consumption have to include complex and
expensive purification procedures to reduce pollutant
levels to acceptable values.

15   The present invention aims to provide an improved process
for increasing the polyunsaturated fatty acid content of
oil compositions, and particularly a process which is well
adapted to a commercial process for recovering EPA and/or
DHA from fish oil in enhanced yield.

20   As is known, lipases are well suited for use as catalysts
in processes involving highly labile n-3 polyunsaturated
fatty acids, such as EPA and DHA, occurring in marine oil.
This is due to their ability to act at low temperatures,
their neutral pH and their mildness in action, which helps
25   keep to a minimum undesired side reactions such as
cis-trans isomerizations, double-bond migrations,
polymerizations, oxidations, etc. Thus, the utilization of
lipases for the hydrolysis of fatty acids in marine oil is
already well documented.

30   For instance, Lie and Lambertsen in Comp. Biochem. Physiol.
**80B** No. 3, pages 447-450, 1985, reported that intestinal
lipase obtained from cod hydrolysed preferentially the
polyunsaturated fatty acids 18:4, 20:5 and 22:6 present as

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triglycerides in capelin oil.   They reported that this
specificity was particularly prominent for the acid 20:5,
i.e. EPA.

5      On the other hand, Lie and Lambertsen also found that the
$C_{14}$-$C_{18}$ saturated and monounsaturated fatty acids were
preferentially hydrolysed as triglycerides from capelin oil
by Candida cylindracea lipase, whereas the long-chain
monoenes,    C20:1    and    C22:1    and    particularly    the
polyunsaturated fatty acids C18:4, and, to a lesser extent
10     EPA, DHA were resistant to hydrolysis (Lie and Lambertsen,
Fette, Seifen, Anstrichmittel, 88, 365, 1986).

A Japanese patent specification No. 59-14793 - Noguchi
et al, describes a method based on similar discrimination
by lipases between saturated and unsaturated acids for
15     preparing concentrates of highly polyunsaturated fatty
acids.     Ethyl esters from miscellaneous marine oils such
as sardine and mackerel oil were hydrolysed with various
lipases (Candida cylindracea, Aspergillus rhizopus and
Mucor miehei).    Selective hydrolysis afforded ethyl ester
20     concentrates of up to 25% EPA and 17% DHA after separation
of the hydrolysed fatty acids.

Another Japanese patent, 172691 - Nippon Oil and Fats, 1990
describes a method based on the hydrolysis of marine oil
with Candida sp. lipase.    EPA or its ester was obtained
25     from a free fatty acid component and DHA or its ester from
the residual glyceride component.    The continuation of the
process involved separation of fatty acid and glyceride
components, esterification with lower alkyl, concentration
of polyunsaturated fatty acids using urea complexion and
30     further purification by molecular distillation, super-
critical $CO_2$ fluid extraction or liquid chromatography.
Takagi (Am. Oil Chem. Soc. 66, 488, 1988) has described a
method based on the reverse process for separating EPA and
DHA using an immobilized Mucor miehei lipase.

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Polyunsaturated fatty acid concentrates obtained from
Japanese sardine oil by the urea adduct method were
esterified with methanol in n-hexane medium at room
temperature. The lipase discriminated between EPA and DHA
5   and selective esterification afforded an EPA enriched
methyl ester concentrate of 51% EPA and 6% DHA as well as
a DHA enriched free fatty acid concentrate of 52% DHA and
12% EPA in the ratio of 59 to 41, respectively.

10   Yamane and co-workers have very recently used several
lipases to selectively hydrolyse cod liver oil and refined
sardine oil (Agric. Biol. Chem. 54, 1459, 1990). The best
results were obtained for the non-regiospecific Candida
cylindracea and 1,3-specific Aspergillus niger lipases, but
none of the lipases were able to raise the EPA content of
15   the glyceride products significantly.

It is apparent from this prior work on the use of lipases
to hydrolyse esters of marine oil fatty acids that
different lipases behave quite differently, and also that
a quite marked selectivity between one fatty acid and
20   another, or between one type of fatty acid and another, is
often exhibited by the lipases.

This substrate selectivity of certain lipases is taken
advantage of by Zuyi and Ward who have described a lipase-
catalysed alcoholysis of cod liver oil to prepare an n-3
25   polyunsaturated fatty acid-enriched fraction (Zuyi and
Ward, "Lipase-catalyzed alcoholysis to concentrate the n-3
polyunsaturated fatty acid of cod liver oil", Enzyme
Microb. Technol., 1993, 15, July, 601-606). The authors
studied nine lipases and they found that Pseudomonas sp.
30   lipase (CES from Amano International Enzyme Co) alcoholized
fish oil at a higher rate than the other lipases and
produced monoglycerides significantly enriched in EPA and
DHA. In this prior process, the alcohol (ethanol and
isopropanol were used) was employed both as a reactant and

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as a solvent for the reaction.

Zuyi and Ward studied the effect of water concentration in
the reaction medium and they found that the alcoholysis
(with isopropanol) by lipase CES increased with water
5    content in the range 0-7.5% v/v.   The data presented shows
that a water content of 5% v/v was optimal, and that even
with a water content of 2.5% v/v over 40% of the original
triglycerides present in the oil remained unreacted after
12 hours (it is understood that these water contents refer
10   to water added to the reaction system and take no account
of the small amounts of water inevitably present in the
fish oil and the "dry" enzyme).   Concomitantly, there was
very considerable hydrolysis of triglyceride to free fatty
acid (typically more than 30%), for instance at a water
15   content of 5% v/v there was 18.9% hydrolysis (compared with
only 15.5% alcoholysis).

Although the Zuyi and Ward process is of scientific
interest it does not, unfortunately, provide an improved
process for preparing purified EPA/DHA compositions
20   commercially.   In particular, the inevitable presence in
the lipase-catalysed product of significant quantities of
free fatty acid make it difficult subsequently to purify
the desired n-3 polyunsaturated fatty acids.   For example,
due to low volatility the free fatty acids cannot be
25   separated from the glycerides by conventional molecular
distillation techniques.    Furthermore, since the esters
and the free fatty acids have substantially different
polarities, whilst the mono- and di-glycerides are of
intermediate polarities, they cannot be separated from the
30   glycerides using extraction.   As demonstrated below, the
present invention in contrast advantageously may use
molecular distillation techniques not only to separate
polyunsaturated fatty acid glycerides from saturated and
monounsaturated    fatty    acid    esters    but    also    to
35   simultaneously effect removal of environmental pollutants

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such as pesticides and polychlorinated biphenyls from the
desired polyunsaturated fatty acid glyceride fraction.

We have now unexpectedly found, in accordance with the
present invention, that certain specific lipases, including
the Pseudomonas sp. lipase used by Zuyi and Ward, can be
used to selectively transesterify the ester moieties of
saturated and monounsaturated fatty acids in marine oil
triglycerides under substantially anhydrous reaction
conditions.    It is found that such transesterification
leads, without substantial concomitant hydrolysis, to a
mixture of monoesters of the more susceptible saturated
fatty acids, with the remaining n-3 polyunsaturated long-
chain fatty acids being still bound to the glycerol moiety
as esters, mainly mono- and diglycerides but also
triglycerides, depending upon the extent of conversion.

More particularly, the present invention provides a process
for treating an oil composition containing saturated and
unsaturated fatty acids in the form of triglycerides, in
order to obtain a refined product with higher
concentrations of polyunsaturated fatty acids, which
process comprises:

        (a)    subjecting the oil composition to a
transesterification reaction with a $C_1$-$C_6$ alcohol under
substantially anhydrous conditions and in the presence of
a lipase active to preferentially catalyze the trans-
esterification of the saturated and monounsaturated fatty
acids, the amount of said $C_1$-$C_6$ alcohol present in the
reaction being not more than 15 molar equivalents based on
the triglycerides present;  and thereafter

        (b)    separating a residual fraction enriched in
glycerides of polyunsaturated fatty acids from a fraction
containing saturated and monounsaturated fatty acid esters
produced by said lipase-catalyzed transesterification

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reaction.

As noted, the process of the present invention utilizes a
lipase which is active to preferentially catalyse the
transesterification of the saturated and monounsaturated
5    fatty acids in the marine oil.   We have found that many
lipases either have relatively poor or indeed no activity
in the transesterification reaction of this invention, or
display a poor selectivity as between saturated and
monounsaturated fatty acids on the one hand and
10   polyunsaturated fatty acids, on the other hand.   This is
true, for instance, of Geotrichum candidum (GCL; Amano GC),
Aspergillus niger (ANL; Amano A), Candida rugosa
(CRL; Amano AY), Chromobacterium viscosum (CVL; Sigma),
Humicula lanuginosa (HLL; Amano CE), Rhizopus delemar
15   (RDL; Amano  D),  Rhizopus  oryzae  (ROL;  Amano  F),
Penicillium camembertii (PCL; Amano G), Candida lipolytica
(CLL; Amano L),  Mucor  javanicus  (MJL;  Amano  M)  and
Rhizopus niveus (RNL; Amano N).    Candida antarctica
(CAL; Novo SP435) was found to be highly active in the
20   transesterification reaction but unfortunately displayed
little or no selectivity between the different classes of
fatty acids and therefore is not suitable for use in the
present invention.

Examples of lipases which we have found are operable in the
25   process of the present  invention include Mucor miehei
lipase (MML; Novo Lipozyme), which displays good trans-
esterification activity and reasonable selectivity as
between polyunsaturated fatty acids and saturated and
monounsaturated  fatty  acids;    Candida  cylindracea
30   (CCL; Sigma) and Penicillium roquefortii (PRL; Amano R)
lipases, both of which exhibit satisfactory selectivity
although their activity is lower than some other lipases;
and  Pseudomonas  fluorescens  (PFL;  Amano  PS)  and
Pseudomonas sp. (PSL; Amano AK) lipases, both of which
35   exhibit good activity and selectivity and therefore are

often preferred for use in the invention.

Immobilisation of the lipase onto a support material may
provide    several    advantages.       For    instance,    the
immobilization gives the lipases higher stability, so they
5   last longer.  It also makes their recovery much easier and
their reuse possible, which dramatically lowers their cost.
Also, the esterification reactions are easier to manipulate
when an immobilized enzyme is involved and the lipase
becomes susceptible to continuous processes which may also
10  be   vital   for   enzymatic   industrialization  processes.
Sometimes, immobilization leads to improvements in the
performance of enzymes.  Finally, the dispersion of the
lipase on the surface of the support material should ensure
the exposure of the lipase to the substrates, leading to
15  dramatic increase in activity of the enzyme per weight unit
and considerable cut in dosage of enzyme and thus the cost
involved.

If it is desired to prepare a refined fish oil composition
enriched with both EPA and DHA then it is preferred to use
20  a lipase which is substantially inactive towards both these
n-3 polyunsaturated fatty acids, i.e. which does not
significantly   discriminate  between   EPA   and   DHA.
Preferably  there  is  used  in  this  case  Pseudomonas
fluorescens and Pseudomonas sp. lipases, the latter being
25  especially preferred.   These lipases are available from
Amano International Enzyme Co of Nagoya, Japan.

It is a critical feature of the present process that the
lipase-catalysed transesterification should be conducted
under   substantially   anhydrous   reaction   conditions.
30  Preferably, the total amount of water in the reaction
system, from all sources including the marine oil and the
lipase, should be less than 1% w/w, preferably less than
0.5% w/w, and most desirably between 0.01-0.25% w/w.
(In a typical case the marine oil starting material will
35  contain 0.1-0.2% w/w water, absolute ethanol used as the

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contain 0.1-0.2% w/w water, absolute ethanol used as the
alcohol reagent will contain 0.2-0.5% w/w water and the
lipase preparation will contain 2-2.5% w/w water.)

However, it is not feasible to employ totally anhydrous
5   reaction conditions since a small amount of water,
generally about 1-2% by weight based on the weight of the
lipase is always required in an enzyme system to establish
activity, but depending very much upon the enzyme in
question (Candida cylindrica lipase, for example, requires
10  the addition of about 10% by weight of water in order to
exhibit optimum activity in the present process).    As
shown in the Examples hereafter, these very small
quantities of water do not lead to any significant
hydrolysis, and it is possible to keep the concentration of
15  free fatty acids in the transesterified product to below 3%
by weight, i.e. only about 10% of the hydrolysis rate
reported by Zuyi and Ward, supra.

Another critical feature of the present invention is that
the amount of alcohol present should be restricted to not
20  more than 15 molar equivalents, preferably not more than 9
molar equivalents, based on the triglycerides present,
(i.e. 3 molar equivalents of alcohol is a stoichiometric
quantity)..    The alcohol thus serves primarily as a
reactant, rather than as a solvent.    It is surprising that
25  the lipase-catalyzed selective transesterification of the
marine oil triglycerides occurs so successfully in such an
essentially solvent-free reaction system.    Preferably, a
substantially stoichiometric quantity of the alcohol is
employed, i.e. from 2 to 5 molar equivalents based on
30  triglycerides present, since amounts of alcohol much in
excess of the stoichiometric quantity lead to a lower
recovery of the desired polyunsaturated fatty acids.

Although it is possible to utilize any $C_1$-$C_6$ alcohol, the
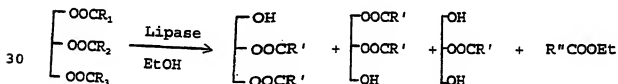use of absolute ethanol (typically with a water content in

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the range of 0.1-0.5% by weight) is preferred, for reasons
of availability and cost, and bearing in mind also the
requirement    for    substantially    anhydrous    reaction
conditions.

5   The temperature at which the transesterification reaction
is conducted is also not critical.   However, it is found
that the selectivity of the reaction decreases with
increasing  temperature,  although  the  reaction  rate
increases.   It is generally preferred to carry out the
10  reaction at a temperature not in excess of 40°-60°C,
depending on the lipase being employed, more preferably at
room temperature (about 20°C).

The    transesterification    may    be    conducted    using
supercritical fluids, e.g. supercritical $CO_2$, as the
15  reaction medium.  For example, ethanolysis of fish oil has
been conducted in supercritical $CO_2$ at 250 bar at 40°C.
The supercritical $CO_2$ fluid can be used not only as a
reaction medium but also to separate the esters and free
fatty acids from the residual glycerides.

20  The lipase-catalyzed transesterification of marine oil
triglycerides in accordance with this invention can be
schematically illustrated by the following, simplified,
reaction equation, using ethanol as the illustrative $C_1$-$C_6$
alcohol.

25  Step (a)

$$
\begin{bmatrix} OOCR_1 \\ OOCR_2 \\ OOCR_3 \end{bmatrix}
\xrightarrow[\text{EtOH}]{\text{Lipase}}
\begin{bmatrix} OH \\ OOCR' \\ OOCR' \end{bmatrix}
+
\begin{bmatrix} OOCR' \\ OOCR' \\ OH \end{bmatrix}
+
\begin{bmatrix} OH \\ OOCR' \\ OH \end{bmatrix}
+ R''COOEt
$$

where $R_1$, $R_2$, $R_3$ indicate the mixed fatty acids (saturated,
monounsaturated  and  polyunsaturated)  present,  as
35      triglycerides, in the original composition

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R' indicates n-3 polyunsaturated fatty acids and

R" indicates saturated and monounsaturated fatty acids

(for simplification only 1,2- and 2,3-diglycerides and
5  2-monoglycerides are shown on the product side).

The separation of the ethyl ester fraction from the
glyceride fraction is suitably carried out by a molecular
distillation technique, whereby the relatively volatile
ethyl esters can be readily removed from the less volatile
10  residual glyceride mixture.   Since the product of the
transesterification reaction contains only small quantities
of free fatty acids, as a consequence of the use of
substantially anhydrous reaction conditions, there can be
obtained following the molecular distillation a residual
15  fraction which is substantially free of the unwanted
saturated and monounsaturated fatty acids.   Even though
the molecular distillation step may result in a small part
of the most volatile monoglycerides appearing in the
distillate, these will be predominantly monoglycerides of
20  relatively short-chain fatty esters (i.e. little or no EPA
or DHA is lost into the distillate).   Similarly a small
part of the ester fraction with low volatility may remain
with the residual glyceride mixture i.e. esters of
predominantly long-chain fatty acids such as EPA and DHA.
25  Accordingly, part of the relatively small amount of the
desired polyunsaturated fatty acids that have participated
in the transesterification will nonetheless remain in the
residual fraction following distillation.   Thus, even
though molecular distillation is not usually regarded as
30  being suitable for difficult separations, in our process it
has proved to be surprisingly advantageous.

Since environmental pollutants such as pesticides and
polychlorinated biphenyls (PCBs) are more volatile than

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glycerides of long chain fatty acids, molecular distillation will remove these compounds from the glyceride fraction whilst they will become concentrated in the distillate (ester fraction). This is yet another
5  advantage of the use of molecular distillation in the present process.

Indeed, in a specific aspect the present invention provides a process for the removal of environmental pollutants from an oil composition containing saturated and unsaturated
10  fatty acids in the form of triglycerides, which process comprises the steps of:

(a) subjecting the oil composition to a trans-esterification reaction with a $C_1$-$C_6$ alcohol under substantially anhydrous conditions and in the presence of
15  a lipase active to preferentially catalyze the trans-esterification of the saturated and monounsaturated fatty acids, the amount of said $C_1$-$C_6$ alcohol present in the reaction being not more than 15 molar equivalents based on the triglycerides present; and thereafter

20  (b) subjecting the product obtained in step (a) to one or more molecular distillations to recover a fraction enriched in glycerides of polyunsaturated fatty acids and from which environmental pollutants have been preferentially removed.

25  The process of the present invention is particularly adapted to the preparation of compositions containing high concentrations, above 40% by weight, preferably above 70% by weight, of EPA and DHA from marine sources. Since the product of the lipase-catalyzed transesterification
30  contains the unwanted saturated and monounsaturated fatty acids predominantly in the form of their ethyl esters (if ethyl alcohol is used) rather than as free acids (the polyunsaturated fatty acids remain substantially as glycerides), the saturated fatty acid fraction can be

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removed by a relatively gentle molecular distillation, with
a relatively low loss of the desired polyunsaturated fatty
acid component.     At the same time, relative volatile
environmental pollutants such as pesticides and PCBs will
5    be removed with the ethyl ester fraction, as discussed
above.        Compared with conventional processes for
manufacturing EPA/DHA concentrates, the present invention,
particularly in its preferred embodiments, thus permits
significant advantages, amongst which can be mentioned:

10            (i)   the absence of any solvent leads to a
considerable reduction in bulkiness, which effect is
enhanced by the ability to use only stoichiometric
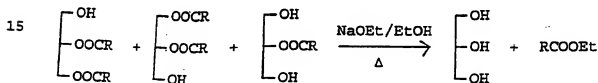concentrations of alcohol,

            (ii)  the transesterification reaction can be
15   conducted under mild e.g. room temperature conditions,
minimizing side reactions and not requiring high energy
inputs,

            (iii) the recovery of EPA and DHA is very high,
and the recovered product is essentially freed of
20   contamination by environmental pollutants, and

            (iv)  the substantially anhydrous reaction
conditions used means that there is minimum hydrolysis,
whereby molecular distillation of the glyceride fraction
from the transesterification reaction gives a good
25   separation of desired polyunsaturated fatty acids from
unwanted saturated and monounsaturated fatty acids.

Thus, the alcoholysis process of the present invention can
be an initial stage in an integrated production process for
the manufacture of an EPA + DHA concentrate.    In such an
30   integrated process, following molecular distillation, or
other technique, to effect the separation of the saturated
fatty acid ethyl ester fraction from the polyunsaturated

fatty acid glyceride fraction, the latter fraction,
containing the desired polyunsaturated fatty acids can be
further treated in order to increase the concentration of
particular acids present. For instance, if it is desired
5    to obtain a highly concentrated EPA- plus DHA-containing
ethyl ester composition then the glyceride fraction
obtained following the molecular distillation can, for
instance, be esterified by chemical transesterification
with absolute ethanol in the presence of a catalytic amount
10   of for example sodium ethoxide or potassium ethoxide.
This process is schematically illustrated by the following
reaction equation:

$$
\begin{bmatrix} \text{OH} \\ \text{OOCR} \\ \text{OOCR} \end{bmatrix}
+
\begin{bmatrix} \text{OOCR} \\ \text{OOCR} \\ \text{OH} \end{bmatrix}
+
\begin{bmatrix} \text{OH} \\ \text{OOCR} \\ \text{OH} \end{bmatrix}
\xrightarrow[\Delta]{\text{NaOEt/EtOH}}
\begin{bmatrix} \text{OH} \\ \text{OH} \\ \text{OH} \end{bmatrix}
+ \text{RCOOEt}
$$

(For simplification, only 1,2- and 2,3-diglycerides and
20   2-monoglycerides are shown above).

The glycerol produced can then be removed using known
techniques. Typically, this will lead to a content of EPA
+ DHA of approximately 45-50% by weight, with a very good
recovery of the polyunsaturated fatty acids, as compared
25   with a conventional process.

More particularly, a preferred integrated process for
obtaining a high concentration EPA + DHA composition in
accordance with the present invention comprises the
following further steps:

30          (c) transesterifying the glyceride fraction with
a lower alcohol, e.g. ethanol, using either chemical
catalysis e.g. an alkaline environment containing amounts
of a base such as sodium or potassium ethoxide sufficient
only to catalyze the transesterification, or enzymatic
35   catalysis e.g. using Candida antarctica lipase under

substantially anhydrous conditions;

(d)   heating the resulting alkyl ester product
with an excess of urea in an alkanol to a temperature of
from 55° to 99°C;

5            (e)   cooling the product of step (d), e.g. to
about 0°-25°C to precipitate urea fatty acid alkyl ester
adduct and thereafter separating off said adduct to leave
a solution mainly containing n-3 fatty acid esters;

(f)   separating from the solution remaining from
10   step (e) the n-3 fatty acid alkyl esters;  and

(g)   removing any solvent from the mixture
obtained in step (f).

In a particularly preferred embodiment of this integrated
process the concentrate obtained in step (g) is further
15   concentrated by means of one or more e.g. nine steps of
molecular distillation in order to increase the EPA plus
DHA concentration therein to 85% by weight, or higher.

Fig.  1  of  the  accompanying  drawings  schematically
illustrates  such  an  integrated  production  process  in
20   accordance with the present invention for the production of
an 85% by weight EPA + DHA ethyl ester concentrate.

As an alternative to chemical transesterification of the
glyceride fraction e.g. with ethanol in the presence of a
catalytic amount of  sodium or potassium ethoxide, this
25   transesterification  may  be  effected  enzymatically  e.g.
using Candida antarctica lipase.   This lipase is quite
active towards n-3 polyunsaturated fatty acids as well as
other fatty acids, and can be employed to effect the trans-
esterification of  the  glyceride  fraction  in  a  highly
30   efficient manner under mild conditions and in the absence

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of solvent, thereby contributing to a further reduction in
bulkiness.

In some instances, it may be desired to isolate a
substantially pure single unsaturated fatty acid from the
5  starting marine oil composition.  Such a process, for the
production of, separately, substantially 100% EPA and 100%
DHA is schematically illustrated in Fig. 2 of the
accompanying drawings.  This embodiment of the invention
utilizes not only a Pseudomonas lipase (PSL) to achieve an
10  initial separation of saturated and unsaturated fatty acids
in accordance with the teachings of this invention, but
also, in subsequent steps Mucor miehei lipase (MML) which
selectively favours the esterification of EPA, rather than
DHA, thereby permitting a good separation of these two
15  acids, and Candida antarctica lipase (CAL) to effect the
ethanolysis of the DHA-enriched glyceride mixture thereby
produced.

More particularly, a preferred integrated process in
accordance with this invention for obtaining substantially
20  pure EPA and substantially pure DHA comprises the following
steps:

       (i)  transesterifying the starting marine oil
with ethanol using PSL under substantially anhydrous
reaction conditions as described above;

25       (ii)  subjecting the lipase-catalyzed trans-
esterification product to molecular distillation to
recover a glyceride fraction containing 40-50% EPA plus
DHA;

       (iii)  transesterifying the glyceride fraction from
30  (ii) with ethanol using the Mucor miehei lipase (MML)
under similar transesterification conditions as for the
initial transesterification (i.e. substantial absence of

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both solvent and water and use of substantially
stoichiometric amounts of the ethanol);

    (iv)  separating the resulting EPA-enriched ethyl
ester fraction and the residual DHA-enriched glyceride
5   mixture by molecular distillation in a similar manner to
step. (ii);

    (v)  working up the EPA-ethyl ester fraction e.g.
by using molecular distillation in combination with urea
precipitation, chromatography etc, to concentrate the EPA
10  fraction to substantially 100% purity;

    (vi)  transesterifying the DHA-enriched glyceride
mixture from the MML mediated transesterification
recovered from step (iii) with ethanol using _Candida
antarctica_ lipase (CAL);  and

15    (vii)  working up the resulting DHA ethyl ester
concentrate by similar techniques to those used in step
(v), to recover substantially 100% pure DHA.

Although it is a particular advantage of the present
invention that it is well adapted to use in integrated
20  processes for the manufacture of compositions having high
concentrations of EPA and/or DHA, it is to be noted that
the same general techniques can be utilized to isolate
other unsaturated fatty acids from marine oil products,
e.g. 18:4 n-3, 20:4 n-3, 21:5 n-3 and 22:5 n-3 fatty acids.
25  The process of the invention is applicable to oil products
containing polyunsaturated fatty acids from other sources
e.g., fermentation products and vegetable and plant oils.
The n-6 polyunsaturated fatty acids are characteristic of
plant and vegetable oils.  n-6 fatty acids of interest
30  include  arachidonic  acid  (AA,  20:4  n-6),  bishomo-γ-

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linolenic acid (BHGLA, 20:3 n-6) and γ-linolenic acid (GLA,
18:3 n-6). Oil products containing arachidonic acid have
also been industrially obtained by fermentation using
Mortierella.

5       It has been found it is readily possible to prepare
compositions containing at least 40% by weight of
arachidonic acid from plant or vegetable oil by the process
of the invention. Preferred lipases for use in the process
are Pseudomonas sp. lipase and Pseudomonas fluorescens
10  lipase. The arachidonic acid fraction may be further
concentrated to substantially 100% purity.

Generally, the marine oil composition which is used as the
starting material in the process of the present invention
may be any raw or partially treated oil from fish or other
15  marine sources and which contains fatty acids, including
polyunsaturated fatty acids, in the form of triglycerides.
Typically, each triglyceride molecule in such a marine oil
will contain, more or less randomly, different fatty acid
ester moieties, be they saturated, monounsaturated or poly-
20  unsaturated, or long chain or short chain.

The present invention is illustrated by the Examples which
follow:

                        Example 1

The purpose of this experiment was to test the use of a
25  number of lipases in a process for separating poly-
unsaturated fatty acids, particularly EPA and DHA, from
saturated and monounsaturated fatty acids in a fish oil
composition.

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The following lipases were tested:

| Name | Abbreviation | State | Supplier |
|------|-------------|-------|----------|
| Mucor miehei | MML | immobilized | Novo |
| Candida antartica | CAL | immobilized | Novo |
| Candida cylindracea | CCL | powder | Sigma |
| Pseudomonas fluorescens | PFL | powder | Amano |
| Penicillium roqueforti | PRL | powder | Amano |
| Pseudomonas sp. | PSL | powder | Amano |

In one test, each of the above lipases was tested at 37°C, and the results are reported in Table I. In another test, the two Pseudomonas lipases, PFL and PSL, were tested at 20°C, and the results are given in Tables II and III.

The procedures used in the tests were as follows:

Fish oil triglycerides were provided by Pronova Biocare a.s., Norway, and contained 14.9% EPA and 9.8% DHA. They were used directly as provided without any further treatment. All solvents were analytical grade and purchased from Merck AG in Germany. Stoichiometric amount of ethanol (absolute) was used, i.e. 3 molar equivalents based on the triglycerides, unless otherwise stated.
Except in the case of using Candida cylindracea lipase, no water was added to the reaction system. It is calculated that the water content, arising from the fish oil triglyceride starting material and the lipase, was of the order of 0.3-0.4% by weight. In the case of Candida cylindricea lipase, water was added in an amount of 10% by weight based on the weight of the lipase, resulting in the reaction system containing about 0.8% by weight water in total, i.e. even in this case substantially anhydrous conditions were maintained.

Fatty acid analyses were performed on methyl esters

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employing a Perkin-Elmer 8140 Gas Chromatograph using a 30
meter capillary column, DB-225 30N 0.25 mm with hydrogen
gas as a carrier gas according to a previously described
procedure (G.G. Haraldsson and Ô. Almarsson, Acta Chemica
5    Scandinavica, 1991, 45, 723-730).      For the detailed
investigation represented in Tables II and III for PSL and
PFL, respectively, preparative thin-layer chromatography
was employed to separate the lipid fractions from the
reaction mixture at appropriate time intervals to monitor
10   the progress of the reactions.     Silica gel plates from
Merck (Art 5721) were used after washing with a 50:50
mixture of chloroform-methanol and heating at 110°C for 30
min.      Elution was performed with 80:20:1 mixture of
petroleum ether-diethyl ether-acetic acid.     Rhodamine 6G
15   (Merck) was used to visualize the bands which subsequently
were scraped off and the fat methylated and analyzed as
quoted above.      Methyl esters of $C_{19,0}$ or $C_{21,0}$, purchased
from Sigma, were added to the samples as internal standards
before injection.

20   In the experiments reported in Table I separation of ethyl
esters and glycerides were performed on a Waters PrepL*
System 500A instrument.  The PrepPak* 500/Silica Cartridge
column from Millipore was eluted with 10% ethyl ether in
petroleum ether at the flow rate of 250 ml/min.     Each run
25   afforded two peaks according to the index of refraction
detector, the first appearing after approximately one
column volume and consisting of pure ethyl esters and the
second one eluting after roughly two column volumes
containing pure triglycerides according to TLC.     The
30   remaining mono- and diglycerides were washed off the column
by methanol.

In a typical procedure the lipase (0.5 g) was added to a
mixture of the fish oil (5.0 g, approximately 5.67 mmol
(M.wt. appr. 882 g/mol)) and absolute ethanol (0.80g, 17.4
35   mmol).   The resulting enzyme suspension was gently stirred

at room temperature (37°C for the experiments in Table I)
under nitrogen.    After appropriate time the reaction was
discontinued by chloroform addition and separation of the
enzyme by filtration.    The organic solvent was removed in
5    vacuo (rotary evaporator and high-vacuum pump treatment) to
afford the crude product mixture in quantitative yields.
The product was dissolved in equal volume of chloroform and
injected into the HPLC instrument as was described above.
Each fraction was collected and the solvent evaporated off
10    in   vacuo,    weighed    and    finally    analyzed    by    gas
chromatography.

When preparative TLC was used instead of preparative HPLC
small samples (100-200 mg) were withdrawn from the reaction
mixture by the aid of a Pasteur pipette at appropriate
15    time.    Enzyme particles were separated by filtering the
sample through a cotton wool plug located inside a second
Pasteur   pipette.    Each   sample   was   then   diluted   in
chloroform  (250 mg/ml)  before  introducing  it  to  the  TLC
plate.

20   The results are presented in Tables I-III below:

The  first  table,  Table  I,  summarizes  the  results  of
experiments conducted at 37°C (using 3 molar equivalents of
ethanol) with all the lipases tested:

Table I

| MML (10%*, 23 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 32.0 | 8.73 | 18.66 | 0.96 | 3.13 |
| TG | 35.0 | 20.12 | 47.04 | 11.49 | 41.14 |
| MG/DG | 32.0 | 16.07 | 34.35 | 17.01 | 55.70 |
| MG/DG/TG | 67.0 | 18.19 | 81.39 | 14.12 | 96.84 |

| CAL (2%*, 10 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 47.3 | 18.51 | 54.51 | 4.33 | 20.36 |
| TG | 33.8 | 15.56 | 32.74 | 12.45 | 41.84 |
| MG/DG | 18.9 | 10.83 | 12.74 | 20.11 | 37.79 |
| MG/DG/TG | 52.7 | 13.86 | 45.49 | 15.20 | 79.64 |

| CAL (2%*, 23 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 69.5 | 17.28 | 76.45 | 6.77 | 44.11 |
| TG | 16.6 | 13.16 | 13.91 | 14.73 | 22.92 |
| MG/DG | 13.9 | 10.90 | 9.64 | 25.30 | 32.97 |
| MG/DG/TG | 30.5 | 12.13 | 23.55 | 19.55 | 55.89 |

| CCL (10%*, 71 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 5 | 6.52 | 2.17 | 2.73 | 1.45 |
| TG | 69 | 16.19 | 74.53 | 10.81 | 79.00 |
| MG/DG | 26 | 13.43 | 23.30 | 7.1 | 19.55 |
| MG/DG/TG | 95 | 15.43 | 97.83 | 9.79 | 98.55 |

| PRL (10%*,102 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 8.4 | 7.70 | 4.12 | 2.48 | 2.14 |
| TG | 61 | 17.08 | 66.37 | 10.53 | 65.90 |
| MG/DG | 30.6 | 15.14 | 29.51 | 10.18 | 31.96 |
| MG/DG/TG | 91.6 | 16.43 | 95.88 | 10.41 | 97.86 |

| PSL (10%*, 5 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 40.3 | 2.42 | 6.00 | 2.92 | 11.70 |
| TG | 11.5 | 30.87 | 21.84 | 13.53 | 15.47 |
| MG/DG | 48.2 | 24.34 | 72.16 | 15.20 | 72.83 |
| MG/DG/TG | 59.7 | 25.60 | 94.00 | 14.88 | 88.30 |

| PFL (10%*, 10 hours) | | EPA | | DHA | |
|---|---|---|---|---|---|
| Fraction | % wt | % comp. | % recovery | % comp. | % recovery |
| EE | 36 | 2.87 | 6.44 | 2.39 | 8.48 |
| TG | 17.7 | 28.23 | 31.13 | 11.41 | 19.90 |
| MG/DG | 46 | 21.78 | 62.43 | 15.8 | 71.62 |
| MG/DG/TG | 63.7 | 23.57 | 93.56 | 14.58 | 91.52 |

*   Percentages refer to the dosage of lipase based on the weight
    of triglycerides

The results for <u>Candida cylindracea</u> (CCL) are with 10%
water added, based on the lipase, since this lipase was
inactive without water addition.   The poor selectivity of
<u>Candida antarctica</u> lipase (CAL) towards EPA and DHA will be
noted, and accordingly this lipase is not useful in this
invention.   In contrast, all the other lipases tested
showed satisfactory activity and selectivity, and the two
<u>Pseudomonas</u> species lipases were outstanding in these
respects.

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## Table II

PSL with 3 molar equivalents of ethanol at 20°C.

Wt % of lipid classes

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|-------|------|------|------|------|------|------|
| MG | 5.0 | 9.4 | 13.0 | 17.9 | 16.8 | 15.9 |
| DG | 29.1 | 35.6 | 31.6 | 23.3 | 24.9 | 25.9 |
| FFA | 2.6 | 2.9 | 2.5 | 2.7 | 2.7 | 2.7 |
| TG | 42.9 | 17.9 | 14.2 | 9.1 | 7.5 | 3.7 |
| EE | 20.4 | 34.2 | 38.7 | 47.0 | 48.1 | 51.8 |

Area % EPA

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|----------|------|------|------|------|------|------|
| MG | 12.0 | 12.8 | 16.4 | 19.2 | 22.5 | 26.1 |
| DG | 19.3 | 21.6 | 26.0 | 28.1 | 32.3 | 32.3 |
| FFA | 3.0 | 2.7 | 5.0 | 6.2 | 5.7 | 9.0 |
| TG | 19.2 | 23.2 | 30.3 | 31.8 | 32.7 | 32.0 |
| EE | 1.1 | 1.2 | 1.6 | 2.0 | 2.2 | 2.8 |
| MG/DG/TG | 18.8 | 20.7 | 24.9 | 25.6 | 29.0 | 30.1 |

Area % DHA

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|----------|------|------|------|------|------|------|
| MG | 15.5 | 16.2 | 16.4 | 17.3 | 18.4 | 17.7 |
| DG | 12.7 | 13.2 | 13.7 | 14.1 | 15.1 | 14.9 |
| FFA | 2.5 | 2.7 | 4.9 | 6.4 | 6.7 | 9.9 |
| TG | 9.8 | 10.3 | 11.6 | 12.2 | 12.9 | 12.3 |
| EE | 1.5 | 1.4 | 1.9 | 2.4 | 2.8 | 3.6 |
| MG/DG/TG | 11.3 | 12.8 | 13.8 | 14.9 | 15.9 | 15.7 |

Area % EPA + DHA

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|---|---|---|---|---|---|---|
| MG | 27.5 | 29.0 | 32.8 | 36.5 | 40.9 | 43.8 |
| DG | 32.0 | 34.8 | 39.7 | 42.2 | 47.4 | 47.2 |
| FFA | 5.5 | 5.4 | 9.9 | 12.6 | 12.4 | 18.9 |
| TG | 29.0 | 33.5 | 41.9 | 44.0 | 45.6 | 44.3 |
| EE | 2.6 | 2.6 | 3.5 | 4.4 | 5.0 | 6.4 |
| MG/DG/TG | 30.0 | 33.6 | 38.7 | 40.5 | 44.9 | 45.8 |

Weight % EPA

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|---|---|---|---|---|---|---|
| MG | 3.5 | 7.8 | 12.1 | 21.4 | 21.8 | 22.4 |
| DG | 37.2 | 56.5 | 53.5 | 51.3 | 53.1 | 51.7 |
| FFA | 0.6 | 0.7 | 0.9 | 1.2 | 1.2 | 1.6 |
| TG | 57.3 | 32.2 | 29.4 | 20.3 | 17.0 | 15.4 |
| EE | 1.5 | 2.9 | 4.1 | 5.7 | 6.9 | 9.0 |
| MG/DG/TG | 98.0 | 96.5 | 95.0 | 93.0 | 91.9 | 89.5 |

Weight % DHA

| Class | 1h | 2h | 4h | 8h | 13h | 24h |
|---|---|---|---|---|---|---|
| MG | 7.5 | 15.6 | 22.8 | 33.0 | 29.9 | 26.2 |
| DG | 40.4 | 55.0 | 48.3 | 38.6 | 41.5 | 41.0 |
| FFA | 0.8 | 1.1 | 1.5 | 2.2 | 2.3 | 3.0 |
| TG | 48.1 | 22.6 | 19.4 | 13.8 | 11.4 | 10.3 |
| EE | 3.3 | 5.7 | 8.0 | 12.4 | 14.8 | 19.6 |
| MG/DG/TG | 96.0 | 93.2 | 90.5 | 85.4 | 82.8 | 77.5 |

Table II above demonstrates that excellent results were obtained with Pseudomonas sp. lipase in the trans-esterification reaction. Thus, 48% conversion was reached after 13 hours leading to 44.9% EPA + DHA content in the glycerides with 91.9% recovery of EPA and 82.8% recovery of DHA. After 24 hours of reaction, 52% conversion was

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obtained leading to 45.8% EPA + DHA content in the
glycerides with 89.5 and 77.5% recovery, respectively, for
EPA and DHA, suggesting that in practice it would be
appropriate to stop the reaction at an earlier stage.
5   The extent of the hydrolysis side reaction remained
constant, and low, throughout the reaction, being in the
range 2.5-2.9%.

## Table III

PFL with 3 molar equivalents of ethanol at 20°C.

10  Wt % of lipid classes

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|-------|------|------|------|------|------|------|------|
| MG | 2.2 | 2.9 | 4.8 | 10.2 | 15.1 | 18.1 | 16.7 |
| DG | 10.8 | 15.6 | 23.4 | 34.8 | 29.2 | 22.5 | 16.0 |
| FFA | 0.9 | 0.9 | 1.3 | 1.2 | 1.2 | 1.0 | 1.4 |
| TG | 81.9 | 71.0 | 49.1 | 27.3 | 15.8 | 9.9 | 6.3 |
| EE | 4.2 | 9.6 | 21.3 | 26.5 | 38.7 | 48.4 | 59.7 |

Area % EPA

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|-------|------|------|------|------|------|------|------|
| MG | 11.6 | 12.7 | 13.7 | 12.7 | 14.5 | 16.9 | 21.5 |
| DG | 17.9 | 19.6 | 16.9 | 17.8 | 25.5 | 28.9 | 27.1 |
| FFA | 3.5 | 4.5 | 6.3 | 5.1 | 5.5 | 8.8 | 14.2 |
| TG | 15.9 | 16.4 | 18.0 | 22.0 | 29.2 | 31.8 | 26.7 |
| EE | 0.0 | 1.5 | 1.3 | 1.9 | 2.2 | 3.1 | 4.7 |
| MG/DG/TG | 16.0 | 16.8 | 17.4 | 18.7 | 23.7 | 25.2 | 24.6 |

Area % DHA

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|---|---|---|---|---|---|---|---|
| MG | 10.5 | 14.7 | 18.1 | 18.1 | 18.6 | 18.7 | 13.2 |
| DG | 12.2 | 12.9 | 13.5 | 10.9 | 13.8 | 14.4 | 11.8 |
| FFA | 3.4 | 3.5 | 4.3 | 4.4 | 3.2 | 9.4 | 8.4 |
| TG | 9.6 | 9.6 | 9.3 | 9.8 | 10.8 | 11.4 | 10.1 |
| EE | 0.0 | 1.3 | 1.1 | 1.5 | 2.0 | 2.7 | 3.7 |
| MG/DG/TG | 9.9 | 10.3 | 11.1 | 11.5 | 14.2 | 15.4 | 12.1 |

Area % EPA + DHA

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|---|---|---|---|---|---|---|---|
| MG | 22.1 | 27.4 | 31.8 | 30.8 | 33.1 | 35.6 | 34.7 |
| DG | 30.1 | 32.5 | 30.4 | 28.7 | 39.3 | 43.3 | 38.9 |
| FFA | 6.9 | 8.0 | 10.6 | 9.5 | 8.7 | 18.2 | 22.6 |
| TG | 25.5 | 26.0 | 27.3 | 31.8 | 40.0 | 43.2 | 36.8 |
| EE | 0.0 | 2.8 | 2.4 | 3.4 | 4.2 | 5.8 | 8.4 |
| MG/DG/TG | 25.9 | 27.2 | 28.5 | 30.2 | 37.9 | 40.5 | 36.8 |

Weight % EPA

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|---|---|---|---|---|---|---|---|
| MG | 1.4 | 2.0 | 4.1 | 8.0 | 12.7 | 18.8 | 25.5 |
| DG | 12.1 | 19.4 | 28.0 | 43.6 | 49.2 | 46.1 | 35.3 |
| FFA | 0.2 | 0.3 | 0.6 | 0.5 | 0.5 | 0.7 | 1.7 |
| TG | 86.3 | 77.5 | 65.4 | 44.4 | 32.2 | 23.7 | 14.4 |
| EE | 0.0 | 0.9 | 1.9 | 3.5 | 5.5 | 10.6 | 23.0 |
| MG/DG/TG | 99.8 | 98.9 | 97.5 | 96.0 | 94.1 | 88.6 | 75.2 |

Weight % DHA

| Class | 1h | 2h | 4h | 8h | 14h | 25h | 49h |
|---|---|---|---|---|---|---|---|
| MG | 2.0 | 3.8 | 8.5 | 18.5 | 27.1 | 33.7 | 28.1 |
| DG | 13.3 | 20.7 | 35.1 | 44.0 | 44.3 | 36.9 | 27.6 |
| FFA | 0.4 | 0.4 | 0.7 | 0.7 | 0.4 | 1.2 | 1.8 |
| TG | 84.3 | 73.9 | 53.2 | 32.5 | 19.9 | 13.5 | 9.8 |
| EE | 0.0 | 1.3 | 2.5 | 4.4 | 8.2 | 14.7 | 32.6 |
| MG/DG/TG | 99.6 | 98.4 | 96.8 | 95.0 | 91.3 | 84.1 | 65.5 |

Table III above shows that good results in the trans-
esterification are also obtained with Pseudomonas
fluorescens lipase, although the rate of conversion is
rather slower than with PSL.    Thus, after 25 hours, 48%
conversion was obtained, which increased to 60% after 49
hours.    On the other hand, the extent of hydrolysis was
somewhat lower.    The performance of this lipase is also
inferior to that of Pseudomonas sp. lipase in terms of EPA
and DHA recovery (88.6% and 84.1%, respectively) and EPA +
DHA composition (40.5%) after 25 hours.

In the above Tables I-III the following abbreviations are
used:

        MG      monoglyceride
        DG      diglyceride
        TG      triglyceride
        FFA     free fatty acid
        EE      ethyl ester
        Area % EPA          Area % is based on the
        Area % DHA          integration of the corresponding
        Area % EPA+DHA      GC-chromatograms

These abbreviations will be used throughout the remaining
Examples.

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It is clear from the above tables that both the Pseudomonas
lipases exhibit uniquely low affinity towards EPA and DHA
but high affinity towards the initial triglycerides.

It will also be noted that the two Pseudomonas lipases to
5      some extent tend to favour the transesterification of DHA,
rather than EPA.   This is most unusual behaviour, since
all other lipases which we have tested show the reverse
tendency.

It will be further noted from Tables II and III that after
10     a certain time the MG/DG/TG ratio starts to decline from
the optimum.   Accordingly, it is normally desirable to
conclude the transesterification reaction before it has
gone to completion.

Example 2

15     This experiment was designed to test the two Pseudomonas
lipases PSL and PFL in larger scale runs.

Example 2a - PSL

Pseudomonas sp. lipase (100 g, 25 200 activity units/g) was
added to a mixture of the fish oil (1000 g, approximately
20     1.13 mol) and absolute ethanol (170 g, 3.70 mol).   The
resulting enzyme  suspension  was  gently  stirred  on  a
magnetic stirrer at room temperature under nitrogen.   The
water content of the reaction mixture was calculated to be
approximately 0.3-0.4% by weight.

25     After 25 hours a sample was withdrawn according to the
procedure  described  in  Example 1  and  analysed.    The
reaction was allowed to continue for another 24 hours.   A
centrifuge was employed for separating the reaction mixture
and the lipase (5000 rpm for 10 min).

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When the reaction was stopped after 50 hours the product had the following composition:

Table IV

| 50 hours | | EPA | | DHA | | EPA + DHA |
|---|---|---|---|---|---|---|
| Lipid Class | % wt | % area | % wt | % area | % wt | % area |
| MG | 17.1 | 17.0 | 18.0 | 17.8 | 28.9 | 34.8 |
| DG | 26.0 | 28.9 | 53.1 | 14.5 | 42.1 | 43.4 |
| FFA | 2.8 | 5.7 | 1.2 | 6.0 | 1.9 | 11.7 |
| TG | 8.9 | 28.8 | 19.3 | 11.8 | 12.2 | 40.6 |
| EE | 46.2 | 2.6 | 8.4 | 2.9 | 14.8 | 5.5 |
| MG/DG/TG | 52.0 | 25.0 | 90.4 | 15.1 | 83.2 | 40.1 |

Part of the product (902.4 g) was degassed under vacuum at 80°C in order to remove volatile components. The volatile components were collected in a cold trap cooled with liquid nitrogen. After degassing 844.1 g of glyceride/ethyl/ ester mixture remained. 756.3 g of this mixture was distilled in a molecular distillation still at 125°C, 0.005 mbar. This gave a distillate of 358.6 g (47.4%) and a residue weighing 388.3 g (51.3%). The fractions had the following composition.

Table V

| | TG A% | DG A% | MG A% | EE A% | EPA A% | DHA A% | EPA+DHA A % |
|---|---|---|---|---|---|---|---|
| after de- gassing | 11.2 | 35.5 | 19.7 | 33.6 | 16.4 | 11.0 | 27.4 |
| disti- llate | - | 0.9 | 10.6 | 88.5 | 3.6 | 3.4 | 7.0 |
| residue | 18.5 | 54.0 | 25.2 | 2.3 | 29.1 | 18.2 | 47.3 |

The table shows that the residue contains 47.3% EPA + DHA,

i.e. a higher concentration of these desired n–3 polyunsaturated fatty acids than prior to the molecular distillation (see Table IV). This means that it is ideal for further concentration using known techniques, i.e. urea
5   fractionation followed by molecular distillation.

It should be noted that the distillate contains appreciable amounts of monoglycerides (10.6%), while the residue contains 2.3% ethyl esters. As discussed above, these will predominantly be monoglycerides from relatively short-
10  chain fatty acids and ethyl esters from long-chain fatty acids, respectively.

### Example 2b

1 kg of the same oil as in Example 2a was transesterified with ethanol using the PFL enzyme. The reaction was
15  stopped after 48 hours. Composition of intermediary product is shown in Table VI.

### Table VI

| 48 hours | | | EPA | | DHA | | EPA + DHA |
|---|---|---|---|---|---|---|---|
| Lipid Class | % wt | % area | % wt | % area | % wt | % area |
| MG | 16.2 | 14.3 | 14.0 | 16.4 | 27.3 | 30.7 |
| DG | 26.8 | 26.5 | 48.6 | 13.7 | 43.1 | 40.2 |
| TG | 14.9 | 26.3 | 28.3 | 9.0 | 17.9 | 36.2 |
| EE | 42.2 | 2.9 | 9.9 | 2.4 | 11.6 | 5.3 |
| MG/DG/TG | 57.9 | 23.0 | 90.9 | 13.5 | 88.3 | 36.5 |

The intermediary product was degassed and molecular distilled under the same conditions as in Example 2a. The results are tabulated below.

| | TG A% | DG A% | MG A% | EE A% | EPA A% | DHA A% | EPA+DHA A % |
|---|---|---|---|---|---|---|---|
| after de-gassing | 15.8 | 31.4 | 20.1 | 32.8 | 15.9 | 10.0 | 25.9 |
| disti-llate | - | - | 13.7 | 86.3 | 4.2 | 2.9 | 7.1 |
| residue | 25.7 | 48.8 | 23.5 | 2.0 | 27.8 | 18.1 | 45.9 |

## Example 3

The products resulting from three pilot plant runs similar to that of Example 2 were analyzed to determine the level of environmental pollutants.   The results are shown in Table VII below:

## Table VII

Results from three examples (mg/kg):

| | $\alpha$-BCH | HCB | total DDT | Toxaphene |
|---|---|---|---|---|
| Starting material | nd | nd | 0.03 | nd |
| PSL glyceride product | nd | nd | nd | nd |
| PSL ethyl ester by-product | nd | 0.01 | 0.03 | nd |
| PFL #1 glyceride product | nd | nd | nd | nd |
| PFL #1 ethyl ester by-product | 0.005 | 0.02 | 0.03 | 0.4 |
| PFL #2 glyceride product | nd | nd | nd | nd |
| PFL #3 ethyl ester by-product | nd | 0.03 | 0.03 | 0.4 |

nd  = not detected
HCH = hexachlorocyclohexane
HCB = hexachlorobenzene
DDT = dichlorodiphenyltrichloroethane

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The above results show that the pollutants are concentrated
in the ethyl ester fractions.  For some of the pesticides,
the levels in the starting material obviously were just
below the detection limit of the analytical method.  This
5    is the reason why we detected these pesticides in the ethyl
ester fractions but not in the original fish oil.
Strangely, the DDT level does not seem to increase in the
ethyl ester fraction compared to the original oil.  Before
we separated the ethyl esters and glycerides (by molecular
10   distillation) we performed a gentle molecular distillation
step in order to strip the reaction mixture of unreacted
ethanol.  Evidently this ethanol stripping was sufficient
to remove part of the DDT.  Analysis of the recovered
ethanol (example PSL) showed total DDT of 0.03 mg/kg, while
15   none of the other pesticides could be detected.  .

### Example 4

The purpose of this experiment was to compare different
procedures for immobilising lipases and to investigate the
effect of using lipases in an immobilised form in the
20   process of the invention.


A.   Immobilisation procedures


(i)    PSL and PFL immobilisation on Duolite
Duolite A 562 (Duolite International; 10.0 g) was
washed 3-4 times with 30 mM NaOH (250 ml each time) on
25   a Buchner funnel and then placed into a 150 ml beaker
together with water (40 ml) and a magnetic follower.
The pH of that solution was adjusted to pH 8 by the aid
of an autotitrator (1.00 M NaOH).  If the initial pH is
below pH 5.0 it may take considerable time to reach
30   equilibrium at pH 8.  If the pH on the other hand is
above pH 8.0 it does not drop until the lipase solution
is added.  The lipase (2.0 g, PSL/PFL) dissolved in
water (20 ml) was added to the Duolite solution via a

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Pasteur pipette. With the aid of the autotitrator the pH was maintained between pH 8.0-8.4 during the addition and the solution was magnetically stirred for 1 hour. After that time 95-99% lipase had been immobilised on the Duolite. After stirring, the lipase preparation was washed with a buffer solution (Tris-HCl; pH 8.0). Finally, the immobilised lipase preparation was dried in vacuo (0.1-0.01 mmHg) for 0.5-1 hour. A steam bath (40°C) may be used to speed up the drying process. The immobilised lipase preparation was stored in a refrigerator (4°C).

(ii)  PSL immobilisation on Amberlite

Amberlite XAD-7 resin (50g; Rohm and Haas; 70% water-content) was thoroughly washed with 0.1 M sodium phosphate buffer at pH 7.0 (twice with 125 ml). PSL powder (Amano AK; 1.5g; 20,000 lipase units (LU, defined as μmol of free fatty acids produced in one minute as based on tributyrin hydrolysis; 17 LU/mg powder) was dissolved in 0.1 M sodium phosphate buffer at pH 7.0 (200 ml). When a drop in pH occurred, pH was adjusted to 7.0. The lipase solution was then added to the resin and the resulting suspension stirred until at least 95% of immobilisation had taken place (about 1 hour) as established by activity measurement of the supernatant liquid by tributyrin hydrolysis.

(iii)   Activity measurement of immobilised lipase
         preparations

The immobilised resin (about 40 mg) was accurately weighed and added to n-butyl palmitate (310-315 mg). To that mixture was added 95% ethanol (120μl) and the mixture stirred well for 90 min. A sample was collected and analyzed by GLC. The amount of lipase alcohol units (LAU) is defined as the amount of ethyl palmitate (μmol) produced in one min.

B.    Productivity experiments with immobilised PSL.
Fish oil ethanolysis productivity experiments for PSL
immobilised on both Duolite and Amberlite were carried
5      out according to the following method.


(i)    Productivity experiments with immobilised PSL
Fish oil (100g), absolute ethanol (20ml) and water (1
ml) were mixed together and the resulting mixture
vigorously stirred until a good dispersion had formed.
10     The moisture-free immobilised lipase (10g) was added to
the dispersion, which was vigorously agitated until a
clear solution had formed.    Then, the mixture was
stirred gently, until a conversion of approximately 50-
% had been reached (24 hours).    The lipase was
15     separated off by filtration and used in the successive
runs, whereas the fat was analysed as described
earlier.

The results of the productivity experiments with PSL
immobilised on Duolite are given in Table VIII.

### Table VIII
Productivity experiments with PSL on Duolite.

| Class | Run 1 | Run 2 | Run 3 | Run 4 | Run 5 | cont. |
|---|---|---|---|---|---|---|
| **Weight % of Lipid Classes** | | | | | | |
| EE | 46.1 | 51.0 | 51.7 | 49.2 | 46.9 | |
| FFA | 4.5 | 4.4 | 5.0 | 4.1 | 5.6 | |
| MG | 15.9 | 16.8 | 17.3 | 16.5 | 17.0 | |
| DG | 26.3 | 22.8 | 21.3 | 25.2 | 25.1 | |
| TG | 7.3 | 5.1 | 4.7 | 5.0 | 5.5 | |
| MG/DG/TG | 49.5 | 44.7 | 43.3 | 46.7 | 47.6 | |
| **Area % EPA** | | | | | | |
| EE | 1.6 | 1.9 | 1.9 | 1.8 | 1.8 | |
| FFA | 7.6 | 8.9 | 9.8 | 6.8 | 6.9 | |
| MG | 26.5 | 26.5 | 25.1 | 23.7 | 22.9 | |
| DG | 42.0 | 37.6 | 37.1 | 35.3 | 35.7 | |
| TG | 41.0 | 35.8 | 35.2 | 35.6 | 42.7 | |
| MG/DG/TG | 36.9 | 33.2 | 32.1 | 31.2 | 31.9 | |
| **Area % DHA** | | | | | | |
| EE | 1.9 | 2.3 | 2.3 | 2.2 | 2.1 | |
| FFA | 9.3 | 10.8 | 9.8 | 8.1 | 18.4 | |
| MG | 20.2 | 20.0 | 19.8 | 18.0 | 18.5 | |
| DG | 17.5 | 15.6 | 15.0 | 14.6 | 14.5 | |
| TG | 15.5 | 12.5 | 13.2 | 12.7 | 13.9 | |
| MG/DG/TG | 18.1 | 16.9 | 16.7 | 15.6 | 15.9 | |
| **Area % EPA+DHA** | | | | | | |
| EE | 3.5 | 4.2 | 4.2 | 4.0 | 3.9 | |
| FFA | 16.9 | 19.7 | 19.6 | 14.9 | 25.3 | |
| MG | 46.7 | 46.5 | 44.8 | 41.7 | 41.4 | |
| DG | 59.6 | 53.2 | 52.1 | 49.8 | 50.2 | |
| TG | 56.5 | 48.4 | 48.3 | 48.3 | 56.5 | |
| MG/DG/TG | 55.0 | 50.1 | 48.8 | 46.8 | 47.8 | |
| **Weight % EPA** | | | | | | |
| EE | 3.8 | 6.1 | 6.4 | 5.7 | 5.1 | |
| FFA | 2.0 | 2.7 | 3.6 | 2.0 | 2.6 | |
| MG | 19.1 | 24.3 | 25.0 | 21.9 | 20.8 | |
| DG | 57.9 | 54.3 | 52.8 | 57.7 | 55.5 | |
| TG | 17.1 | 12.6 | 12.2 | 12.7 | 16.0 | |
| MG/DG/TG | 94.2 | 91.2 | 90.0 | 92.3 | 92.3 | |
| **Weight % DHA** | | | | | | |
| EE | 8.7 | 13.2 | 14.1 | 12.7 | 10.6 | |
| FFA | 4.7 | 5.9 | 6.3 | 4.4 | 12.1 | |
| MG | 27.9 | 32.7 | 34.2 | 30.7 | 29.1 | |
| DG | 46.4 | 40.3 | 37.5 | 43.8 | 39.2 | |
| TG | 12.4 | 7.9 | 8.0 | 8.4 | 9.0 | |
| MG/DG/TG | 86.7 | 80.8 | 79.6 | 82.9 | 77.3 | |

## Table VIII continued
Productivity experiments with PSL on Duolite.

| Class | Run 6 | Run 7 | Run 8 | Run 9 | Run 10 |
|---|---|---|---|---|---|
| **Weight % of Lipid Classes** | | | | | |
| EE | 55.4 | 53.7 | 54.9 | 48.3 | 48.9 |
| FFA | 2.5 | 2.3 | 3.0 | 3.6 | 4.4 |
| MG | 16.5 | 14.7 | 13.5 | 16.7 | 14.6 |
| DG | 23.7 | 27.4 | 26.1 | 25.5 | 25.9 |
| TG | 1.8 | 1.9 | 2.5 | 5.9 | 6.2 |
| MG/DG/TG | 42.0 | 44.0 | 42.1 | 48.1 | 46.7 |
| | | | | | |
| **Area % EPA** | | | | | |
| EE | 1.7 | 1.9 | 1.6 | 1.4 | 1.4 |
| FFA | 11.2 | 11.8 | 11.1 | 8.2 | 7.7 |
| MG | 22.4 | 19.5 | 17.4 | 20.9 | 21.6 |
| DG | 35.1 | 36.7 | 34.2 | 36.3 | 38.7 |
| TG | 29.1 | 31.7 | 34.8 | 44.4 | 42.6 |
| MG/DG/TG | 29.9 | 30.7 | 30.0 | 32.0 | 33.9 |
| | | | | | |
| **Area % DHA** | | | | | |
| EE | 1.9 | 2.2 | 1.8 | 1.5 | 1.6 |
| FFA | 18.8 | 20.6 | 21.5 | 9.0 | 7.7 |
| MG | 15.4 | 16.3 | 14.9 | 17.6 | 18.9 |
| DG | 13.2 | 15.0 | 14.1 | 15.1 | 16.1 |
| TG | 9.8 | 10.7 | 11.7 | 14.2 | 14.0 |
| MG/DG/TG | 13.9 | 15.3 | 14.2 | 15.9 | 16.7 |
| | | | | | |
| **Area % EPA+DHA** | | | | | |
| EE | 3.6 | 4.1 | 3.4 | 2.9 | 3.0 |
| FFA | 30.0 | 32.4 | 32.6 | 17.2 | 15.4 |
| MG | 37.8 | 35.7 | 32.1 | 38.5 | 40.5 |
| DG | 48.3 | 51.7 | 48.3 | 51.4 | 54.7 |
| TG | 38.9 | 42.4 | 46.6 | 58.7 | 56.6 |
| MG/DG/TG | 43.8 | 45.8 | 44.2 | 47.9 | 50.6 |
| | | | | | |
| **Weight % EPA** | | | | | |
| EE | 7.1 | 7.0 | 6.8 | 4.1 | 4.1 |
| FFA | 2.3 | 2.1 | 2.8 | 2.0 | 2.2 |
| MG | 23.9 | 17.0 | 15.3 | 18.7 | 16.4 |
| DG | 62.4 | 69.3 | 67.9 | 57.4 | 60.0 |
| TG | 4.4 | 4.6 | 7.3 | 17.7 | 17.4 |
| MG/DG/TG | 90.6 | 90.9 | 90.5 | 93.8 | 93.7 |
| | | | | | |
| **Weight % DHA** | | | | | |
| EE | 15.0 | 14.4 | 13.1 | 8.8 | 8.9 |
| FFA | 7.3 | 6.5 | 9.6 | 4.2 | 4.2 |
| MG | 30.7 | 25.4 | 23.4 | 30.3 | 27.7 |
| DG | 44.2 | 50.9 | 49.7 | 45.7 | 48.1 |
| TG | 2.8 | 2.8 | 4.4 | 10.9 | 11.1 |
| MG/DG/TG | 77.7 | 79.2 | 77.4 | 87.0 | 86.8 |

Table VIII demonstrates that a consistently high degree of
conversion (46-55%) was obtained in ten successive runs
thereby establishing the productivity of the immobilised
lipase.  The recovery of EPA and DHA was consistently at
least as good or better than that demonstrated in Table II
for the lipase powder, even though only a fifth of the
amount of lipase was used.  Accordingly, the EPA plus DHA
content in the glyceride product mixture remained very high
indeed, ranging from 44-55%.  The extent of the hydrolysis
side reaction was slightly higher than in Table II,
presumably as a result of the higher water content required
for the immobilised lipase, although the water content of
the reaction was still below the stipulated limit of 1%.

The results of the productivity experiments with PSL
immobilised on Amberlite under exactly the same reaction
conditions as for the Duolite case are given in Table IX.

## Table IX

Productivity experiments with PSL on Amberlite.

| Run | % Conversion | EPA | | | DHA | | |
|---|---|---|---|---|---|---|---|
| | | Ar. % EE | Wt. % EE | Ar. % GL | Ar. % EE | Wt. % EE | Ar. % GL |
| 1 | 48.2 | 2.3 | 6.6 | 30.1 | 3.1 | 15.0 | 16.3 |
| 2 | 48.6 | 2.5 | 7.5 | 29.6 | 3.1 | 16.2 | 15.2 |
| 3 | 49.2 | 2.4 | 7.4 | 29.4 | 3.2 | 16.6 | 16.4 |
| 4 | 47.6 | 2.6 | 7.1 | 30.8 | 3.2 | 15.6 | 15.7 |
| 5 | 45.7 | 2.6 | 6.5 | 31.6 | 3.4 | 14.2 | 17.3 |
| 6 | 48.8 | 2.7 | 7.8 | 30.7 | 3.5 | 16.8 | 16.8 |
| 7 | 48.5 | 2.6 | 7.4 | 30.4 | 3.4 | 16.8 | 15.7 |
| 8 | 50.1 | 2.8 | 8.6 | 30.1 | 3.0 | 16.7 | 15.3 |

(Abbreviations:  Ar. % = area %; GL = glyceride product =
MG/DG/TG.).

It is clear from Table IX that the immobilisation on
Amberlite was even more successful that the one on Duolite.
The amount of immobilised lipase is only one-half that
required .on Duolite (one-tenth of the original amount of
lipase powder) to obtain similar results.    Thus,   a
consistent extent of conversion (48 - 50%) was obtained in
eight successive runs after 24 hours reaction time with the
EPA + DHA composition of the glyceride product mixture
consistently remaining between 45 and 47 %. The EPA and DHA
recovery remained  similar  as  did  the  extent  of  the
hydrolysis side-reaction (water content below the stipulated
limit of 1%, but results of hydrolysis not included in the
table) as compared to the Duolite preparation.

### Example 5

The purpose of this experiment was to assess the activity of
Candida antarctica lipase (CAL) and Mucor miehei lipase
(MML) in the ethanolysis of a glyceride mixture produced by
transesterifying a fish oil in the presence of PSL, as
previously described.

(a)      CAL ethanolysis of glycerides
Immobilised CAL (Novo-Nordisk, SP 435, 1-2% water-content;
0.5 g) was added to a glyceride mixture (2.5g; approximately
8.5 mmol of ester equivalents; initial composition; 25.0 %
EPA   and   15.1   %   DHA,   produced   by   PSL-catalysed
transesterification of a fish oil) and absolute ethanol
(0.80 g, 17.4 mmol).   The resulting enzyme suspension was
gently stirred at room temperature under nitrogen. Samples
were  collected  at  appropriate  times  and  analysed  as
previously described.   The reaction was discontinued by
separating off the enzyme by filtration after 22 hours.

The results are given in Table X below.

TABLE X

The progress of the ethanolysis of glyceride mixture with
CAL at 20°C (EE product).

| Time (min/hour) | 10 min | 20 min | 30 min | 40 min | 50 min |
|---|---|---|---|---|---|
| Wt. % EE | 12.7 | 20.3 | 29.1 | 29.0 | 35.8 |
| % A EPA | 29.0 | 29.3 | 30.2 | 34.5 | 29.4 |
| Wt. % EPA | 15.4 | 24.6 | 36.8 | 42.6 | 34.2 |
| % A DHA | 5.7 | 6.3 | 6.9 | 6.9 | 7.2 |
| Wt. % DHA | 5.4 | 9.5 | 14.7 | 14.7 | 14.7 |

cont.

| Time (min/hour) | 1 hour | 3 hours | 6 hours | 10 hours | 22 hours |
|---|---|---|---|---|---|
| Wt. % EE | 40.2 | 68.1 | 76.6 | 80.4 | 100.0 |
| % A EPA | 28.1 | 25.5 | 27.0 | 26.0 | 25.3 |
| Wt. % EPA | 47.1 | 72.6 | 83.4 | 83.9 | 98.0 |
| % A DHA | 7.1 | 8.2 | 10.3 | 10.4 | 14.8 |
| Wt. % DHA | 20.8 | 40.4 | 55.4 | 54.9 | 100.0 |

Table X demonstrates the progress of the ethanolysis of a
glyceride mixture with CAL at 20°C. Approximately a two-
fold excess of ethanol was used as based on mol equivalents
of ester groups present in the initial glyceride mixture.
Only the composition of the ethyl ester product is shown
in the table.   It can be seen that the reaction had
proceeded to completion after 22 hours reaction time.
Also, although the lipase displays high activity toward
both EPA and DHA, it is evident that it displays
consideraly higher activity toward the former fatty acid,
that, is EPA.

(b)       MML ethanolysis of glycerides

The reaction conditions were identical to those of the CAL
ethanolysis reaction described in (a) above.  Immobilised
MML (Novo-Nordisk, Lipozyme™, 10% water-content; 0.5g) was
added to the glyceride mixture (2.5g; approximately 8.5
mmol of ester equivalents;
initial composition:  25 % EPA and 15.1 % DHA, produced by
PSL-catalysed  transesterification  of  a  fish  oil)  and
absolute ethanol (0.80 g, 17.4 mmol).  The resulting enzyme
suspension was gently stirred at room temperature under

nitrogen. Samples were collected at appropriate
times and analysed as previously described. The reaction
was discontinued by separating off the enzyme by filtration
after 27 hours.

The progress of the ethanolysis of the same glyceride
sample as used in the CAL reaction with MML at 20°C is
demonstrated in Table XI below.

Table XI

The progress of the ethanolysis of glyceride mixture with
MML at 20°C.

| Time | 1h | 2h | 3h | 6h | 9h | 12h | 27h |
|---|---|---|---|---|---|---|---|
| % Conv. | 15.8 | 21.7 | 25.4 | 33.7 | 39.5 | 43.0 | 50.1 |
| Ethyl esters: | | | | | | | |
| % Area EPA | 25.4 | 28.7 | 30.4 | 32.1 | 31.5 | 32.3 | 28.4 |
| % (w/w) EPA | 14.4 | 24.1 | 27.5 | 39.3 | 47.1 | 50.2 | 55.0 |
| % Area DHA | 3.7 | 2.9 | 3.7 | 5.0 | 5.5 | 6.6 | 6.3 |
| % (w/w) DHA | 4.0 | 4.7 | 6.4 | 11.2 | 15.2 | 16.7 | 21.7 |
| Residual MG/DG/TG | | | | | | | |
| % area EPA | 26.4 | 26.5 | 30.1 | 23.1 | 21.8 | 20.8 | 20.9 |
| % (w/w) EPA | 85.6 | 75.9 | 72.5 | 60.7 | 52.9 | 49.8 | 45.0 |
| % area DHA | 18.3 | 20.9 | 23.2 | 23.2 | 23.0 | 22.9 | 24.9 |
| % (w/w) EPA | 96.0 | 95.7 | 93.6 | 88.8 | 86.8 | 83.3 | 78.3 |

It is evident from Table XI that MML displays a good degree
of discrimination between EPA and DHA in the ethanolysis
reaction of the EPA/DHA enriched glycerides. At 50%
conversion, after 27 hours reaction time, about 80% of the
initial DHA content remains in the residual glyceride
mixture. The distribution of EPA was not quite as
favourable, 55 % in the ethyl esters and still 45 % in the
glyceride residue. This may be improved by running a
second ethanolysis reaction on the glyceride residue under
similar conditions. It should also be noted that the
activity of MML provides a possibility of using EPA-

enriched fish oil for producing EPA and DHA-enriched fish
oil (e.q. tuna oil) for producing DHA by this methodology.

## Example 6

The purpose of this experiment was to demonstrate the
applicability of the process of the invention to plant and
vegetable oils.

### PSL-catalysed ethanolysis of arachidonic acid extract (AA)

An arachidonic acid (AA, 20:4 n-6) extract, provided by
Pronova Biocare, containing 31.1% AA was treated according
to the method described in Example 1 for fish oil, that is,
10% PSL as powder, 20°C, 50% conversion, 24 hours.

The results are given in Table XII below.

### Table XII

| Class | Wt. % class | Area % AA | Wt. % AA |
|-------|-------------|-----------|----------|
| EE | 50 | 7.6 | 12 |
| FFA | 2 | 23.3 | 1 |
| MG | 17 | 49.0 | 24 |
| DG | 24 | 57.4 | 47 |
| TG | 8 | 58.9 | 16 |
| MG/DG/TG | 49 | 54.2 | 87 |

Table XII demonstrates a very high recovery of AA in the
glyceride mixture, 87% at 50 % conversion, higher than DHA,
but lower than EPA in the corresponding fish oil reactions.
The extent of the hydrolysis side-reaction was comparable
to that in the fish oil experiments, 2 %.  The AA content
of the glyceride produce mixture was 54.2 %.  Surprisingly,
it was highest in the TG fraction and lowest in the MG
fraction.

CLAIMS:

1.    A process for treating an oil composition containing
saturated and unsaturated fatty acids in the form of
triglycerides, in order to obtain a refined product with
higher concentrations of polyunsaturated fatty acids, which
process comprises:

       (a)   subjecting the oil composition to a trans-
esterification reaction with a $C_1$-$C_6$ alcohol under
substantially anhydrous conditions and in the presence
of a lipase active to preferentially catalyze the trans-
esterification of the saturated and monounsaturated
fatty acids, the amount of said $C_1$-$C_6$ alcohol present in
the reaction being not more than 15 molar equivalents
based on the triglycerides present;  and thereafter

       (b)   separating a residual fraction enriched in
glycerides of polyunsaturated fatty acids from a
fraction containing saturated and monounsaturated fatty
acid esters produced by said lipase-catalyzed trans-
esterification reaction.

2.    A process according to claim 1, wherein said lipase
is in an immobilised form.

3.    A process according to any preceding claim, wherein
said $C_1$-$C_6$ alcohol is absolute ethanol.

4.    A process according to any preceding claim, wherein
the amount of said $C_1$-$C_6$ alcohol is from 2 to 5 molar
equivalents based on the triglyceride present.

5.    A process according to any preceding claim, wherein
said transesterification reaction is carried out at room
temperature.

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6.    A process according to any preceding claim, wherein said separation step (b) comprises one or more molecular distillations.

7.    A process according to any preceding claim, wherein said oil composition is a marine oil composition or a fermentation product containing n-3 polyunsaturated fatty acids.

8.    A process according to any of claims 7, wherein said lipase is one which is substantially inactive towards both EPA and DHA.

9.    A process according to Claim 8, wherein said lipase is Pseudomonas sp. lipase or Pseudomonas fluorescens lipase.

10.    A process according to any one of claims 7 to 9, for the preparation of a composition containing at least 40% by weight of EPA plus DHA comprising the further steps of:

(c)    transesterifying the glyceride fraction with a lower alcohol in an alkaline environment using chemical catalysis, or by enzymatic catalysis using lipase,

(d)    heating the resulting alkyl ester product with an excess of urea in an alkanol to a temperature of from 55°C to 99°C;

(e)    cooling the product of step (d) to precipitate urea fatty acid alkyl ester adduct and thereafter separating off said adduct to leave a solution mainly containing n-3 fatty acid esters;

(f)    separating from the solution remaining from step (e) the n-3 fatty acid alkyl esters; and

(g) removing any solvent from the mixture obtained in step (f).

11. A process according to Claim 10, wherein the concentrate obtained in step (g) is further concentrated by means of one or more steps of molecular distillation in order to increase the concentration of EPA plus DHA therein to 85% or higher.

12. A process according to any one of claims 7 to 9, for obtaining substantially pure EPA and substantially pure DHA comprising the further steps of:

(i) transesterifying the glyceride fraction with a $C_1$-$C_6$ alcohol under substantially anhydrous conditions and in the presence of a lipase active to preferentially catalyze the esterification of EPA;

(ii) separating the resulting EPA-enriched $C_1$-$C_6$ alcohol ester fraction and the residual DHA-enriched glyceride mixture:

(iii) working up the EPA-$C_1$-$C_6$ alcohol ester fraction and concentrating the EPA fraction to substantially 100% purity;

(iv) transesterifying the DHA-enriched glyceride mixture with a $C_1$-$C_6$ alcohol in the presence of a lipase active to catalyse the esterification of DHA; and

(v) working up the resulting DHA-$C_1$-$C_6$ alcohol ester fraction and concentrating the DHA fraction to substantially 100% pure DHA.

13. A process according to claim 12 wherein the lipase used in step (i) is _Mucor_ _miehei_ lipase.

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14.    A process according to claim 12 or claim 13 wherein the lipase used in step (iv) is <u>Candida</u> <u>antarctica</u> lipase.

15.    A process according to any one of claims 12 to 14 wherein the $C_1$-$C_6$ alcohol in steps (i) and/or (iv) is ethanol.

16.    A process according to any one of claims 1 to 6 wherein said oil composition is a plant or vegetable oil composition or a fermentation product containing n-6 polyunsaturated fatty acids.

17.    A process according to claim 16, wherein said lipase is one which is substantially inactive towards arachidonic acid (AA).

18.    A process according to claim 17, wherein said lipase is <u>Pseudomonas</u> <u>sp.</u> lipase or <u>Pseudomonas</u> <u>fluorescens</u> lipase.

19.    A process according to any one of claims 16 to 18, for the preparation of a composition containing at least 40% by weight of arachidonic acid comprising the further steps of:

        (c)    transesterifying the glyceride fraction with a lower alcohol in an alkaline environment using chemical catalysis, or by enzymatic catalysis using lipase.

        (d)    heating the resulting alkyl ester product with an excess of urea in an alkanol to a temperature of from 55°C to 99°C;

        (e)    cooling the product of step (d) to precipitate urea fatty acid alkyl ester adduct and thereafter separating off said adduct to leave a solution mainly containing n-6 fatty acid esters;

(f)    separating from the solution remaining from step (e) the n-6 fatty acid alkyl esters; and

(g)    removing any solvent from the mixture obtained in step (f).

20.  A process according to claim 19, wherein the concentrate obtained in step (g) is further concentrated by means of one or more steps of molecular distillation in order to increase the concentration of arachidonic acid therein to 85% or higher.

21.  A process according to Claim 20 wherein the arachidonic acid fraction is concentrated to substantially 100% purity.

22.  A process for the removal of environmental pollutants from an oil composition containing saturated and polyunsaturated fatty acids in the form of triglycerides, which process comprises the steps of:

(a)  subjecting the oil composition to a trans-esterification reaction with a $C_1$-$C_6$ alcohol under substantially anhydrous conditions and in the presence of a lipase active to preferentially catalyze the trans-esterification of the saturated and monounsaturated fatty acids, the amount of said $C_1$-$C_6$ alcohol present in the reaction being not more than 15 molar equivalents based on the triglycerides present;  and thereafter

(b)  subjecting the product obtained in step (a) to one or more molecular distillations to recover a fraction enriched in glycerides of polyunsaturated fatty acids and from which environmental pollutants have been preferentially removed.

23.  A process according to Claim 21, wherein said step (a) is conducted as defined in any one of Claims 2 to 5.
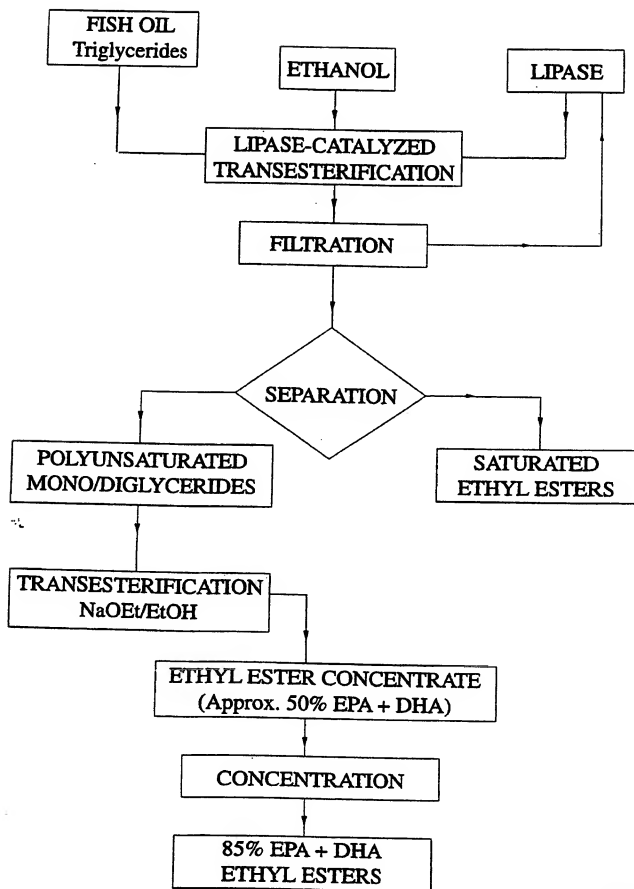
FIG.1

THE PRODUCTION OF 85% EPA+DHA
ETHYL ESTER CONCENTRATE

FIG.2

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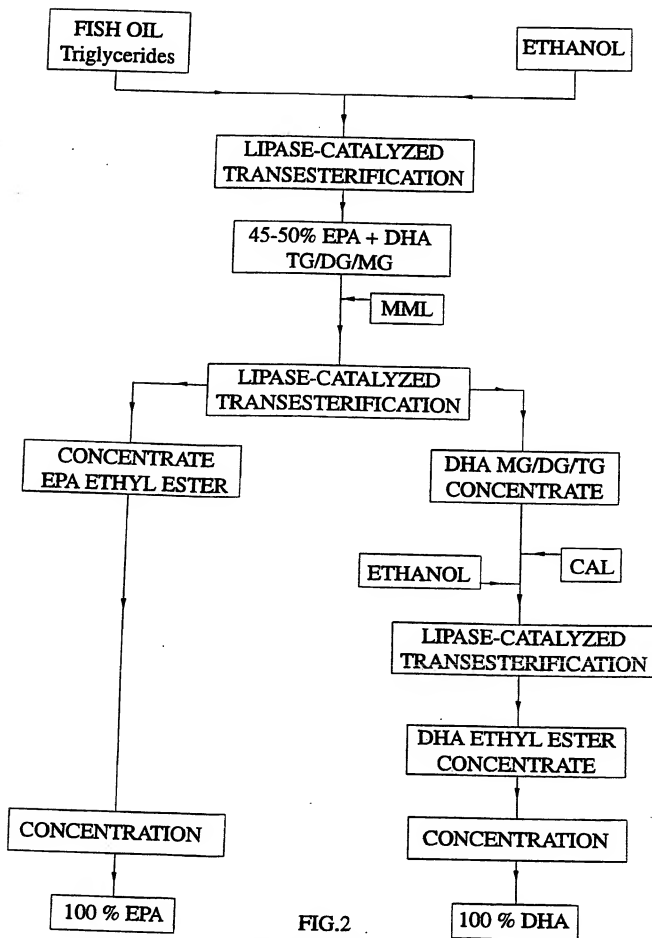| A. CLASSIFICATION OF SUBJECT MATTER |
| --- |
| IPC6: C11C 3/02, C11C 3/06, C07C 69/58, C12P 7/62, C12P 7/64<br>According to International Patent Classification (IPC) or to both national classification and IPC |

| B. FIELDS SEARCHED |
| --- |
| Minimum documentation searched (classification system followed by classification symbols) |
| IPC6: C11C, C07C, C12D |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched |
| SE,DK,FI,NO classes as above |
| Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) |
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| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
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| ☒ Further documents are listed in the continuation of Box C. | ☒ See patent family annex. |
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| * Special categories of cited documents:<br>"A" document defining the general state of the art which is not considered to be of particular relevance<br>"E" earlier document but published on or after the international filing date<br>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>"O" document referring to an oral disclosure, use, exhibition or other means<br>"P" document published prior to the international filing date but later than the priority date claimed | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>"&" document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
| --- | --- |
| 14 June 1995 | 1 6 -06- 1995 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br><br>Eva Johansson<br>Telephone No. +46 8 782 25 00 |

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